HILL TOP RESEARCH, INC.

APPENDIX III

NEUTRALIZER

NEUTRALIZER/TOXICITY EVALUATION TEST

PURPOSE

To determine the effectiveness of a neutralizer to inactivate an antimicrobial agent and to determine if the neutralizer is toxic to the test organism.

TEST ARTICLES

The test articles, identified by the sponsor as 3554-194 and 3554-196, were received from Dial Corporation for use in this test.

NEUTRALIZER

The neutralizer used for this test was D/E Neutralizing Broth.

Neutralizer/Toxicity Blanks, test articles, control ingredients and test organisms used in this neutralization study were as follows:

1) One (1.0) mL of diluted test articles (75% in sterile purified water).

2) A 1.0 mL portion of the test organism suspensions, further diluted in AOAC Phosphate Buffer Dilution Water to approximately 1 x 10^4 CFU/mL was used to deliver ~ 1 x 10^2 CFU/mL in the final dilution for the test organisms, *Enterococcus faecalis*, ATCC 29212, *Staphylococcus aureus*, ATCC 6538, *Corynebacterium minutissimum* ATCC 23347 and *Streptococcus pneumoniae*, ATCC 6303.

3) Ninety-nine (99) mL of D/E Neutralizing Broth.

4) Ninety-nine (99) mL of AOAC Phosphate Buffer Dilution Water.

PROCEDURE

The procedure used for this test is outlined in the Protocol and the following modifications are a result of the particular test organisms, neutralizer, and test conditions.

The test organisms used for this study were *Enterococcus faecalis*, ATCC 29212, *Staphylococcus aureus*, ATCC 6538, *Corynebacterium minutissimum* ATCC 23347 and *Streptococcus pneumoniae*, ATCC 6303. They were prepared as outlined in the protocol.

Neutralizer Effectiveness

One bottle of neutralizer and one bottle of AOAC Phosphate Buffered Water were equilibrated to 20-25°C and then 1.0 mL of the diluted test article was added and thoroughly mixed. Immediately following mixing, 1.0 mL of the test organism (adjusted to ~1 x 10^4 CFU/mL) was added to each bottle and mixed again. Immediately following this second mixing, three 1.0 mL aliquots and three 0.1 mL aliquots, from each bottle, were plated. The plates were incubated at 35 ± 2°C for 48 ± 2 hours. Following incubation, colony forming units (CFU's) per mL were calculated. This procedure was repeated after 20 minutes from the same bottles.

Neutralizer Toxicity

One bottle of neutralizer and one bottle of AOAC Phosphate Buffered Water were equilibrated to 20-25°C and then 1.0 mL of the test organism (adjusted to ~1 x 10^4 CFU/mL) was added. Immediately following mixing, three 1.0 mL aliquots and three 0.1 mL aliquots, from each bottle, were plated.

Neutralizer Toxicity (Cont.)

The plates were incubated at 35 ± 2°C for 48 ± 2 hours. Following incubation, colony forming units (CFU's) per mL were calculated. This procedure was repeated after 20 minutes from the same bottles.

Neutralizer toxicity is evident if more than a 50% difference is observed in recovery of numbers of test organism in the neutralizer used versus recovery from the AOAC Phosphate Buffer Dilution Water. Similarly, neutralizer effectiveness is evident if less than a 50% difference is observed in numbers of test organism recovered when comparing counts in the neutralizer with and without the test substance added.

RESULTS

Results of both neutralizer toxicity and neutralizer effectiveness are shown in the attached Tables of Results on pages 35 - 43.

CONCLUSION

The neutralizer was effective in neutralizing the diluted test articles (75%) and was not toxic to the test organisms.

TABLE I-A OF RESULTS
NEUTRALIZER/TOXICITY RESULTS

Test Article: 3554-194
Test Date: July 31, 2003
Test Organism: Co*rynebacterium minutissimum*, ATCC 23347

Neutralizer Effectiveness

Neutralizer Medium				AOAC Phosphate Buffer Water			
0 Time		20 Minutes		0 Time		20 Minutes	
1.0 mL	0.1 mL	1.0 mL	0.1 mL	1.0 mL	0.1 mL	1.0 mL	0.1 mL
86	1	86	5	77	0	25	4
61	3	62	4	60	6	0	6
95	8	61	5	76	2	8	3
Avg. Count	81	Avg. Count	70	Avg. Count	71	Avg. Count	11
Adjusted Count*	80	Adjusted Count*	69	Adjusted Count*	70	Adjusted Count*	11

Neutralizer Toxicity

Neutralizer Medium				AOAC Phosphate Buffer Water			
0 Time		20 Minutes		0 Time		20 Minutes	
1.0 mL	0.1 mL	1.0 mL	0.1 mL	1.0 mL	0.1 mL	1.0 mL	0.1 mL
73	4	75	2	LA	4	64	9
60	9	90	5	LA	7	91	4
85	8	78	6	LA	5	53	6
Avg. Count	73	Avg. Count	81	Avg. Count	53	Avg. Count	69

Underlined values used in calculations
LA = Lab Accident; probable plating error
* Average Count is multiplied by a 0.99 conversion factor for comparing Neutralizer and Toxicity Test results

TABLE I-B OF RESULTS
NEUTRALIZER/TOXICITY RESULTS

Test Article: 3554-196
Test Date: July 31, 2003
Test Organism: Cor*ynebacterium minutissimum*, ATCC 23347

Neutralizer Effectiveness

Neutralizer Medium				AOAC Phosphate Buffer Water			
0 Time		20 Minutes		0 Time		20 Minutes	
1.0 mL	0.1 mL	1.0 mL	0.1 mL	1.0 mL	0.1 mL	1.0 mL	0.1 mL
105	10	49	2	0	0	0	0
86	7	126	4	0	0	0	0
96	6	69	4	0	0	0	0
Avg. Count	96	Avg. Count	81	Avg. Count	<1	Avg. Count	<1
Adjusted Count*	95	Adjusted Count*	80	Adjusted Count*	<1	Adjusted Count*	<1

Neutralizer Toxicity

Neutralizer Medium				AOAC Phosphate Buffer Water			
0 Time		20 Minutes		0 Time		20 Minutes	
1.0 mL	0.1 mL	1.0 mL	0.1 mL	1.0 mL	0.1 mL	1.0 mL	0.1 mL
73	4	75	2	LA	4	64	9
60	9	90	5	LA	7	91	4
85	8	78	6	LA	5	53	6
Avg. Count	73	Avg. Count	81	Avg. Count	53	Avg. Count	69

Underlined values used in calculations
LA = Lab Accident; probable plating error
* Average Count is multiplied by a 0.99 conversion factor for comparing Neutralizer and Toxicity Test results

TABLE II-A OF RESULTS
NEUTRALIZER/TOXICITY RESULTS

Test Article: 3554-194
Test Date: July 25, 2003
Test Organism: *Enterococcus faecalis*, ATCC 29212

Neutralizer Effectiveness

Neutralizer Medium								AOAC Phosphate Buffer Water							
0 Time				20 Minutes				0 Time				20 Minutes			
1.0 mL			0.1 mL	1.0 mL			0.1 mL	1.0 mL			0.1 mL	1.0 mL			0.1 mL
26	31	28	13	34	39	30	10	26	25	34	8	29	33	18	7
22	32	34	14	32	32	26	10	18	47	29	22	39	35	37	8
21	34	26	10	35	31	35	7	28	36	46	12	22	25	38	7
Avg. Count			85	Avg. Count			98	Avg. Count			96	Avg. Count			92
Adjusted Count*			84	Adjusted Count*			97	Adjusted Count*			95	Adjusted Count*			91

Neutralizer Toxicity

Neutralizer Medium								AOAC Phosphate Buffer Water							
0 Time				20 Minutes				0 Time				20 Minutes			
1.0 mL			0.1 mL	1.0 mL			0.1 mL	1.0 mL			0.1 mL	1.0 mL			0.1 mL
38	28	32	8	42	34	47	15	45	42	47	12	42	36	52	12
32	30	40	8	31	21	42	15	37	37	45	16	37	43	32	6
29	23	32	17	37	37	32	14	37	45	36	14	58	35	46	19
Avg. Count			95	Avg. Count			108	Avg. Count			124	Avg. Count			127

Underlined values used in calculations
1.0 mL - 1 mL spread across 3 plates, counts totaled
* Average Count is multiplied by a 0.99 conversion factor for comparing Neutralizer and Toxicity Test results

TABLE II-B OF RESULTS
NEUTRALIZER/TOXICITY RESULTS

Test Article: 3554-196
Test Date: July 25, 2003
Test Organism: *Enterococcus faecalis*, ATCC 29212

Neutralizer Effectiveness

Neutralizer Medium								AOAC Phosphate Buffer Water							
0 Time				20 Minutes				0 Time				20 Minutes			
1.0 mL			0.1 mL	1.0 mL			0.1 mL	1.0 mL			0.1 mL	1.0 mL			0.1 mL
14	17	19	9	27	28	24	9	30	32	39	13	24	32	36	5
21	13	25	14	36	31	25	11	26	37	39	9	32	38	30	18
18	43	25	13	39	33	23	15	28	25	24	9	20	35	29	11
Avg. Count			65	Avg. Count			89	Avg. Count			93	Avg. Count			92
Adjusted Count*			64	Adjusted Count*			88	Adjusted Count*			92	Adjusted Count*			91

Neutralizer Toxicity

Neutralizer Medium								AOAC Phosphate Buffer Water							
0 Time				20 Minutes				0 Time				20 Minutes			
1.0 mL			0.1 mL	1.0 mL			0.1 mL	1.0 mL			0.1 mL	1.0 mL			0.1 mL
38	28	32	8	42	34	47	15	45	42	47	12	42	36	52	12
32	30	40	8	31	21	42	15	37	37	45	16	37	43	32	6
29	23	32	17	37	37	32	14	37	45	36	14	58	35	46	19
Avg. Count			95	Avg. Count			108	Avg. Count			124	Avg. Count			127

Underlined values used in calculations
1.0 mL - 1 mL spread across 3 plates, counts totaled
* Average Count is multiplied by a 0.99 conversion factor for comparing Neutralizer and Toxicity Test results

TABLE III-A OF RESULTS
NEUTRALIZER/TOXICITY RESULTS

Test Article: 3554-194
Test Date: July 24, 2003
Test Organism: *Staphylococcus aureus*, ATCC 6538

Neutralizer Effectiveness

Neutralizer Medium				AOAC Phosphate Buffer Water			
0 Time		20 Minutes		0 Time		20 Minutes	
1.0 mL	0.1 mL	1.0 mL	0.1 mL	1.0 mL	0.1 mL	1.0 mL	0.1 mL
76	8	89	12	57	6	65	10
55	7	79	16	52	6	52	15
65	9	82	9	59	9	60	7
Avg. Count 65		Avg. Count 83		Avg. Count 56		Avg. Count 59	
Adjusted Count* 64		Adjusted Count* 82		Adjusted Count* 55		Adjusted Count* 58	

Neutralizer Toxicity

Neutralizer Medium				AOAC Phosphate Buffer Water			
0 Time		20 Minutes		0 Time		20 Minutes	
1.0 mL	0.1 mL	1.0 mL	0.1 mL	1.0 mL	0.1 mL	1.0 mL	0.1 mL
78	10	82	8	62	20	60	1
79	9	78	8	72	9	78	5
71	8	93	10	73	7	89	6
Avg. Count 76		Avg. Count 84		Avg. Count 69		Avg. Count 76	

Underlined values used in calculations
* Average Count is multiplied by a 0.99 conversion factor for comparing Neutralizer and Toxicity Test results

TABLE III-B OF RESULTS
NEUTRALIZER/TOXICITY RESULTS

Test Article: 3554-196
Test Date: July 24, 2003
Test Organism: *Staphylococcus aureus*, ATCC 6538

Neutralizer Effectiveness

Neutralizer Medium				AOAC Phosphate Buffer Water			
0 Time		20 Minutes		0 Time		20 Minutes	
1.0 mL	0.1 mL	1.0 mL	0.1 mL	1.0 mL	0.1 mL	1.0 mL	0.1 mL
64	6	83	6	0	0	0	5
60	10	71	5	0	0	0	4
71	8	80	9	0	0	0	0
Avg. Count 65		Avg. Count 78		Avg. Count <1		Avg. Count <1	
Adjusted Count* 64		Adjusted Count* 717		Adjusted Count* <1		Adjusted Count* <1	

Neutralizer Toxicity

Neutralizer Medium				Phosphate Buffer Water			
0 Time		20 Minutes		0 Time		20 Minutes	
1.0 mL	0.1 mL	1.0 mL	0.1 mL	1.0 mL	0.1 mL	1.0 mL	0.1 mL
78	10	82	8	62	20	60	1
79	9	78	8	72	9	78	5
71	8	93	10	73	7	89	6
Avg. Count 76		Avg. Count 84		Avg. Count 69		Avg. Count 76	

Underlined values used in calculations
* Average Count is multiplied by a 0.99 conversion factor for comparing Neutralizer and Toxicity Test results

TABLE IV-A OF RESULTS
NEUTRALIZER/TOXICITY RESULTS

Test Article: 3554-194
Test Date: July 24, 2003
Test Organism: *Streptococcus pneumoniae*, ATCC 6303

Neutralizer Effectiveness

Neutralizer Medium								AOAC Phosphate Buffer Water							
0 Time				20 Minutes				0 Time				20 Minutes			
1.0 mL			0.1 mL	1.0 mL			0.1 mL	1.0 mL			0.1 mL	1.0 mL			0.1 mL
19	24	6	18	23	21	8	5	44	44	42	11	42	48	56	7
18	14	13	8	20	15	20	4	52	53	62	13	49	31	39	16
25	15	17	17	14	17	19	15	56	46	53	14	59	29	50	17
Avg. Count			50	Avg. Count			52	Avg. Count			151	Avg. Count			134
Adjusted Count*			50	Adjusted Count*			51	Adjusted Count*			146	Adjusted Count*			133

Neutralizer Toxicity

Neutralizer Medium								AOAC Phosphate Buffer Water							
0 Time				20 Minutes				0 Time				20 Minutes			
1.0 mL			0.1 mL	1.0 mL			0.1 mL	1.0 mL			0.1 mL	1.0 mL			0.1 mL
19	24	23	12	28	23	21	10	48	36	42	16	59	49	42	22
34	31	26	16	12	34	21	15	46	45	48	16	49	42	51	19
20	24	27	11	18	27	17	11	48	44	39	25	39	46	48	24
Avg. Count			76	Avg. Count			67	Avg. Count			132	Avg. Count			142

Underlined values used in calculations
1.0 mL - 1 mL spread across 3 plates, counts totaled
* Average Count is multiplied by a 0.99 conversion factor for comparing Neutralizer and Toxicity Test results

TABLE IV-B OF RESULTS
NEUTRALIZER/TOXICITY RESULTS

Test Article: 3554-196
Test Date: July 24, 2003
Test Organism: *Streptococcus pneumoniae*, ATCC 6303

Neutralizer Effectiveness

Neutralizer Medium								AOAC Phosphate Buffer Water							
0 Time				20 Minutes				0 Time				20 Minutes			
1.0 mL			0.1 mL	1.0 mL			0.1 mL	1.0 mL			0.1 mL	1.0 mL			0.1 mL
16	12	19	16	17	11	13	11	37	27	47	18	29	55	42	13
12	31	20	12	17	14	19	12	44	34	49	17	35	49	45	10
12	30	22	10	23	19	15	14	37	23	4	10	36	59	34	24
Avg. Count			58	Avg. Count			49	Avg. Count			101	Avg. Count			128
Adjusted Count*			57	Adjusted Count*			48	Adjusted Count*			100	Adjusted Count*			127

Neutralizer Toxicity

Neutralizer Medium								AOAC Phosphate Buffer Water							
0 Time				20 Minutes				0 Time				20 Minutes			
1.0 mL			0.1 mL	1.0 mL			0.1 mL	1.0 mL			0.1 mL	1.0 mL			0.1 mL
19	24	23	12	28	23	21	10	48	36	42	16	59	49	42	22
34	31	26	16	12	34	21	15	46	45	48	16	49	42	51	19
20	24	27	11	18	27	17	11	48	44	39	25	39	46	48	24
Avg. Count			76	Avg. Count			67	Avg. Count			132	Avg. Count			142

Underlined values used in calculations
1.0 mL - 1 mL spread across 3 plates, counts totaled
* Average Count is multiplied by a 0.91 conversion factor for comparing Neutralizer and Toxicity Test results